Remarks

Upon entry of the foregoing amendment, claims 88-90, 98, 105, 109, 11 6-1 19, 160, 163, 164 and 167 are pending in this application. Claims 99-1 04, 106-1 08, 11 0- 1 15, 127-1 30, 138-1 41, 149-1 52, 1 62, 166, 168, 170, 171 , 173, 174, 176, 177, 179, 180, 182, 183 and 185-1 99 have been withdrawn from consideration as being directed to a non-elected invention. Claims 1-87, 91 -97, 120-1 26, 131 -1 37, 142-1 48, 153-1 59, 161, 165, 169, 172, 175, 178, 181 and 184 have been previously canceled without prejudice or disclaimer of the canceled subject matter. Applicant maintains the right to file one or more continuation or divisional applications on any canceled subject matter.

The Claimed Invention

The pending claims are drawn to a composition consisting of 3-O-deacylated monophosphoryl lipid A or monophosphoryl lipid A in combination with granulocyte macrophage colony stimulating factor (GM-CSF), together with a diluent or carrier that can be used in the form of a stable oil-in water emulsion. This adjuvant-cytokine formulation is used to enhance the immune response in a vertebrate host to an antigen, wherein the antigen is derived from a pathogenic virus, particularly polypeptides, peptides or fragments derived from the human immunodeficiency virus (HIV).

Additionally, methods are claimed to elicit a CTL immune response by administering said composition to a host wherein the immune response elicits cytotoxic T- lymphocytes (CTL).

The invention described herein discloses that the combination of an antigen, a selected cytokine, and an immunostimulating complex lipid adjuvant, MPL, increases the immune response specific for the antigen. The invention is exemplified in a model system using peptide antigens derived from HIV. The claimed antigen-cytokine-adjuvant combination induces high titers of antigen-specific and virus neutralizing antibody and also induces good cellular responses as determined through induction of CTL.

Claim Rejections -35 USC §103(a)

Claims 88-90 stand rejected under 35 USC §103(a) as allegedly being obvious over Ulrich et al. (Vaccine Design. Plenum Press, New York, N.Y., pg. 495-523, hereafter "Ulrich") and Disis et al. (Blood, 1996; Vol.88, No.1:202-210,hereafter "Disis"). The Examiner contends that Ulrich taught that the immunostimulant MPL delivered in aqueous admixtures, in oil and water emulsions, or in liposomal vehicles, has adjuvant activity. The Examiner also asserts that Disis taught that GM-CSF is an effective adjuvant. It is the Examiner's opinion that both Ulrich and Disis teach adjuvant compositions that would allegedly be prima facie obvious to yield Applicant's claimed invention.

Claims 88, 98 and 116-119 stand rejected under 35 USC §103(a) as allegedly being obvious over Ulrich and Disis as applied to claim 88, in view of Bartlett et al. (hereafter "Bartlett). Bartlett is cited for teaching the same amino acid sequence as SEQ ID NO:2 of the present invention to elicit an HIV-antigen specific response. The Examiner contends that it would have been prima facie obvious for one skilled in the art at the time the invention was made to combine the adjuvant composition of Ulrich and Disis with the HIV antigen of Bartlett.

Claims 88, 98, 105, 109, 116, 160, 163-164 and 167 stand rejected under 35 USC §103(a) as allegedly being obvious over Ulrich and Disis in view of Bartlett, as applied to claims 88, 98 and 116. The Examiner asserts the opinion that in addition to the previous rejections regarding Applicant's claimed composition that the administration of the antigen in Bartlett would necessarily induce a CTL response in the subject because the antigen of Bartlett contains CTL specific epitopes and would necessarily induce a CTL response. Applicant respectfully disagrees and traverses these rejections.

A key aspect of all three sets of rejections listed above is the Examiner's conclusion that MPL and GM-CSF are art-recognized equivalents ("hence in accordance with *In re Kerkhoven* and MPEP §2144.06, it is prima facie obvious to combine two compositions each of which is taught by the prior art to be useful for the same purpose", page 4 of the Office Action dated June 28, 2007). The Examiner contends that because MPL and GM-CSF are both allegedly "adjuvants", it would have been obvious to one of ordinary skill in the art to combine them into one composition to enhance the immune

response against an antigen of interest. Applicant respectfully disagrees and traverses these rejections.

Ulrich discloses that the immunostimulant complex lipid MPL delivered in aqueous admixtures, in oil-in-water emulsions, or in liposomal vehicles, has adjuvant activity. Nowhere in this reference do the authors teach or suggest that the immune response to the disclosed combination of antigen and MPL could be enhanced by the addition of an immunomodulator, specifically GM-CSF. Disis discloses GM-CSF to be an effective immunomodulator, but without any teaching or suggestion to combine GM-CSF with any other adjuvant or cytokine to enhance an immune response to an antigen.

The Applicant submits that there is nothing in Ulrich or Disis that would prompt the skilled artisan to combine an antigen with the claimed adjuvant combination of MPL and GM-CSF. The Examiner does not point to specific information in Disis that suggests its combination with Ulrich to yield the claimed invention. Instead, the Examiner merely notes how the two references **can** be combined to read on the claimed invention without any explanation as to **why** the skilled artisan would be motivated to combine them. This reference-by reference, limitation-by-limitation analysis wholly fails to demonstrate how the cited references teach or suggest the combination claimed in the present invention. As stated in *Bausch & Lomb v. Barnes-Hind/Hydrocurve, Inc., 230* USPQ 416, 419 (Fed. Cir. 1986), "It is impermissible within the framework of section 103 to pick and choose from any one reference only so much of it as will support a given position to the exclusion of other parts necessary to the full appreciation of what such reference fairly suggests to one skilled in the art."

In lieu of an explanation as to why one of skill in the art would be motivated to combine the references, the Examiner instead cites *In re Kerkhoven* and MPEP §2144.06 in support of this rejection. *In re Kerkhoven,* and the related section of the MPEP, stand for the proposition that it is prima facie obvious to combine two compositions, each of which are taught by the prior art, to form a third composition which is useful for the same purpose. MPEP §2144.06, "Art Recognized Equivalents for the Same Purpose: Combining Equivalent Known for the Same Purpose.", *In re Kerkhoven* presented a case where the claims were related to a process of preparing a "new" spray-dried detergent by mixing two conventional spray-dried detergents together. The new combination was held as being prima facie obvious based on two equivalent

compositions being added together to form a third composition useful for the same purpose.

In relying on *In re Kerkhoven*, the Examiner concludes that the Applicant's invention is the combination of two compositions that are art-recognized equivalents ("Furthermore, in accordance with *In re Kerkhoven* and MPEP §2144.06, it is prima facie obvious to combine two compositions each of which is taught by the prior to be useful for the same purpose.", pg. 4 of the Office Action dated June 28, 2007). Applicant strongly disagrees.

The skilled artisan would immediately recognize that a cytokine (GM-CSF) and a complex lipid (MPL) are not "art-recognized equivalent" compositions. The skilled artisan would understand, as demonstrated in Exhibit 1 (Virgil EJC Schijn, "Immunological Concepts of Vaccine Adjuvant Activity", Current Opinion in Immunology 2000, 12: 456-463, additionally submitted as an Information Disclosure Statement, hereafter known as "Schijns"), that adjuvants are defined as a group of diverse heterogeneous compounds used to evoke or increase an immune response to an antigen. Adjuvants belong to a class of compositions that would broadly be defined as "any molecule or substance that is able to favor or amplify a particular situation in the cascade of immunological events". Schijns continues by pointing out, on Table 1 on page 458, the different functional categories that are used to classify adjuvants with different activities. For example, some classes of adjuvants localize an antigen in the lymph node where there is a facilitation of antigen uptake and presentation by antigen presenting cells (APCs). Other adjuvants have a depot effect whereby there is a prolonged antigen presentation at the injection site. Yet other adjuvants, such as MPL, cause an increase in signaling of pathogen recognition receptors (PRR) on APCs, which then direct innate immune cells to respond to the antigen. These are only a few examples of the different categories of adjuvants. Clearly, adjuvants are a very broad class of molecules whose function, mechanism and characteristics differ greatly.

As shown in Exhibit 2, cytokines are described as molecules that have very specific functions (Chapter 12 "Cytokines" from Cellular and Molecular Immunology, 2nd edition; pages 240-241, additionally submitted as an Information Disclosure Statement). Cytokines are defined by their individual functions, which are broken down into

categories, namely: 1) mediators of natural immunity; 2) regulators of lymphocyte activation; growth and differentiation; 3) regulators of immune mediated inflammation; and 4) stimulators of immature leukocyte growth and differentiation. GM-CSF, in particular, was known to act on bone marrow progenitor cells already committed to develop into leukocytes, as discussed on page 259. *Id.*

Clearly, complex lipid adjuvants and cytokines are different classes of compositions and are not art-recognized equivalents. It is respectfully submitted that the Examiner has inappropriately equated the broad concepts of immune modulation and adjuvants to assert that all adjuvants and cytokines are art-recognized equivalents. Based on the fact that the Applicant's claimed invention is not the mixing of two equivalents, the Applicant respectfully submits that the Examiner's reliance on MPEP §2144.06 and *In re Kerkhoven* is therefore improper.

Based on the foregoing, it is respectfully submitted that claims 88-90 are not obvious in view of the cited references and withdrawal of this rejection is requested.

As noted above, claims 88, 98 and 116-119 additionally stand rejected under 35 USC §103(a) as allegedly being obvious over Ulrich and Disis as applied to claim 88, in view of Bartlett. Bartlett is cited for teaching the same amino acid sequence as SEQ ID NO:2 of the present invention to elicit an HIV-antigen specific response. It is the Examiner's opinion that it would have been prima facie obvious for one skilled in the art at the time the invention was made to combine the adjuvant composition of Ulrich and Disis with the HIV antigen of Bartlett. The applicant respectfully disagrees and traverses the rejection.

The Examiner has further cited Bartlett to reject the claims that are limited to an HIV peptide. Bartlett merely evaluates the immunogenicity of polyvalent HIV envelope synthetic peptide immunogen in the presence of one adjuvant (incomplete Freund's adjuvant). The peptide taught in Bartlett, C4-V3, has the same amino acid sequence set forth in Applicant's SEQ ID NO:2. The combination of Ulrich and Disis, as already stated, does not render the claimed invention obvious and adding Bartlett's observations on a particular peptide does not change this result. There is nothing in the cited reference to suggest to the skilled artisan that the adjuvant combination of MPL and GM-CSF would have been obvious alone with or without the addition the particular peptide taught in

Bartlett. Applicant respectfully submits that the Examiner's rejection is invalid and should be withdrawn.

Based on the foregoing, it is respectfully submitted that claims 88, 98 and 116-119 are not obvious in view of the cited references and withdrawal of this rejection is requested.

As noted above claims 88, 98, 105, 109, 116, 160, 163-164 and 167 additionally stand rejected under 35 USC §103(a) as allegedly being obvious over Ulrich and Disis in view of Bartlett, as applied to claims 88, 98 and 116. It is the Examiner's opinion that, in addition to the previous rejections regarding Applicant's claimed composition, the administration of the antigen in Bartlett would necessarily induce a CTL response in the subject, because the antigen of Bartlett contains CTL specific epitopes and would necessarily induce a CTL response. Applicant respectfully disagrees and traverses the rejection.

As previously discussed, the combination of teachings from Ulrich and Disis in view of Bartlett does not render the claimed invention obvious. Since independent claim 88 is not rendered obvious by the combination of cited references, dependent claims 98, 105, 109, 1 16, 160, 163, 164 and 167 are likewise not obvious. Applicant respectfully submits that the Examiner's rejection is invalid and should be withdrawn.

Selection of the combination of adjuvants, cytokines and particular peptides is far from routine. Applicant's claimed adjuvant combination elicits high titers and CTL responses, as shown in the specification. The skilled artisan at the time of the invention would not have been able to predict this result. Applicants have previously noted that Boon et al. (WO 98/57659, hereafter "Boon") states that GM-CSF added to a combination of MPL and QS21 was "unable to enhance the effect of the QS21/MPL adjuvant". Boon is concerned with identifying the cytokine that would best augment the already known adjuvant combination of MPL and the saponin QS-21. Boon teaches that GM-CSF does not enhance the effect of an adjuvant formulation that comprises MPL adjuvant. The skilled artisan upon reading Boon would have been compelled to avoid addition of GM-CSF with MPL.

The Examiner noted that the adjuvant formulation of Boon comprises more ingredients than those being claimed and rendered obvious by the cited art. The Examiner followed by citing Kerkhoven and MPEP §2144.06, as already discussed. The

Examiner's rationale appears to suggestthat the combination of **any** adjuvants, cytokines or a mix of adjuvants, cytokines and peptides, as in the Applicant's invention, should therefore work in combination. The Examiner asserts that all of these compositions are equivalents and that any combination should work to enhance an immune response to an antigen. Applicant would like to point out that if, as the Examiner states, that all of these compositions are indeed equivalents and would be obvious to combine to form a third composition used for the same purpose, then Boon's addition of GM-CSF to a MPL/QS21 adjuvant combination should have enhanced the immune response. GM-CSF did not enhance the response. The Examiner has underscored Applicant's argument that not all combinations of adjuvants will work in combination to enhance the immune response to an antigen.

Based on the foregoing, it is respectfully submitted that claims 88, 98, 105, 109, 116, 160, 163-164 and 167 are not obvious in view of the cited references and withdrawal of this rejection is requested.

Applicant respectfully submits that the Examiner has failed to articulate why it would have been apparent to one skilled in the art to combine the elements recited in the pending claims. Most, if not all, inventions arise from a combination of old elements. *See In re Rouffet,* 149 F.3d 1350, 1357, 47 USPQ2d 1453,1457 (Fed. Cir. 1998). Thus, every element of a claimed invention may often be found in the prior art. *Id.* However, identification in the prior art of each individual part claimed is insufficient to defeat patentability of the whole claimed invention. *Id.* Rather, to establish obviousness based on a combination of elements disclosed in the prior art, there must be some motivation, suggestion or teaching of the desirability of making the specific combination that was made by the applicant. *See In re Dance,* 160 F.3d 1339, 1343, 48 USPQ2d 1635, 1637 (Fed. Cir. 1998); *In re Gordon,* 733 F.2d 900, 902, 221 USPQ 1125, 1127 (Fed. Cir. 1984).

The motivation, suggestion or teaching may be found in explicit or implicit teachings within the references themselves, from the ordinary knowledge of those skilled in the art, or from the nature of the problem to be solved. *See WMS Gaming, Inc. v. International Game Tech.,* 51 USPQ2d 1385, 1397 (Fed. Cir. 1999). However, there still must be evidence that "a skilled artisan, confronted with the same problems as the inventor and with no knowledge of the claimed invention, would select the elements from

the cited prior art references for combination in the manner claimed." *In re Rouffet,* 47 USPQ2d at 1456; *see also In re Kotzab,* 55 USPQ2d 1313, 1317 (Fed. Cir. 2000) ("[a] rejection cannot be predicated on the mere identification ... of individual components of claimed limitations. Rather, particular findings must be made as to the reason the skilled artisan, *with no knowledge of the claimed invention,* would have selected these components for combination in the manner claimed.").

Additionally, MPEP 2143.01 IV clearly points out that "a statement that modification of the prior art to meet the claimed invention would have been 'well within the ordinary skill of the art at the time the claimed invention was made' because the references contained all aspects of the claimed invention were individually known in the art is not sufficient to establish a *prima facie* case of obviousness without some objective reason to combine the teaching of the reference." *Ex parte Levengood*, 28 USPQ2d 1300 (Bd. Pat. App. & Inter. 1993) See also In *re Kotzab*, 217 F. 3d 1365, 1371, 55 USPQ2d 1313, 1318 (Fed. Cir. 2000).

On October 10, 2007, examination guidelines for determining obviousness under 35 USC §103 in view of the Supreme Court decision in *KSR International v. Teleflex Inc.* were outlined in the Federal Register (Vol. 72, No. 195). As stated on pg. 57528, middle column, fifth paragraph "Office personnel must explain why the difference(s) between the prior art and the claimed invention would have been obvious to one of ordinary skill in the art". Also on the same page, third column, last paragraph it states "the key to supporting any rejection under 35 USC §103 is the clear articulation of the reason(s) why the claimed invention would have been obvious. The Supreme Court in KSR noted that the analysis supporting a rejection under 35 USC §103 should be made explicit. The Court, quoting *In re Kahn*, stated that 'rejections on obviousness cannot be sustained by mere conclusory statements; instead there must be some articulated reasoning with some rationale underpinning to support the legal conclusion of obviousness" KSR, 550 U.S at ___, 82 USPQ2d at 1396. In KSR, the Court went on to note that "It can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in a way the claimed new invention." *See id.*

Assuming, *arguendo,* that the cited references do teach the individual elements of Applicant's claimed invention, the skilled artisan would not have been able to predict

that the claimed combination of MPL and GM-CSF, without hindsight and having Applicant's specification in hand, would have enhanced the immune response to an antigen. There are no teachings or suggestions to combine the individual elements anywhere in the cited references. According to the Supreme Court in the KSR decision and the Examination Guidelines published in the Federal Register, the Examiner is required to show **why** there would be motivation to combine these individual elements. The Examiner has failed to explain why the skilled artisan would have found it obvious to combine the MPL as taught by Ulrich with the GM-CSF as taught by Disis with the HIV antigen taught by Bartlett.

In summary, the Examiner improperly applies *In re Kerkhoven* and MPEP §2144.06 by taking the broad terms "adjuvant" and "cytokine" and concluding that these two different elements of Applicant's claimed invention are art-recognized equivalents when clearly they are not. Additionally, the Examiner has failed to establish a *prima facie* case of obviousness by failing to meet the standard of the KSR decision and the USPTO's guidelines. The Examiner has also failed to articulate why someone skilled in the art would have predicted the utility of the combination of elements in Applicant's claimed invention. In view of the foregoing, Applicant submits that the Examiner has not established a prima facie case of obviousness. The rejections, therefore, are improper and should be withdrawn.

Conclusion

In conclusion, this reply is believed to be a full response to the outstanding Office Action. Should any issues remain outstanding or if there are any questions concerning this paper, or the application in general, the Examiner is invited to telephone the undersigned representative at the Examiner's earliest convenience.

Respectfully submitted,

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Exhibit 1

Immunological concepts of vaccine adjuvant activity

Commentary

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0952-7915/00/$ – see front matter

Abbreviations

APC	antigen-presenting cell
CTL	cytotoxic T lymphocyte
DC	dendritic cell
hsp	heat-shock protein
ISCOM	immunostimulating complex
LDC	lymphoid DC
LPS	lipopolysaccharide
MDC	myeloid DC
PAMP	pathogen-associated microbial pattern
PRR	pathogen-recognition receptor

Introduction

The successful elimination of pathogens following prophylactic immunization depends to a large extent on the ability of the host's immune system to recognize when it is necessary to become activated and how to respond most effectively, preferably with minimal injury to healthy tissue. In the design of effective, nonreplicating vaccines, immunological adjuvants serve as critical components — other than the antigens — which instruct and control the selective induction of the appropriate type of antigen-specific immune response. Although vaccine adjuvants are recognized as a group of most powerful immunomodulatory agents, little is known about the mechanisms underlying their activity.

This article discusses both well-known and very recent paradigms of immune induction that are likely to explain vaccine adjuvant activity at the cellular and molecular level. As key immunological events it addresses the influence of adjuvants on the delivery of vaccine antigens to particular lymphoid tissues over time, as well as adjuvants' influence on the activation status of antigen-presenting cells (APCs). In addition, this article attempts to categorize the known types of immunological adjuvants according to these concepts. Future investigations addressing these paradigms may enlighten the secrets of immune induction and prove helpful in the rational design of vaccines.

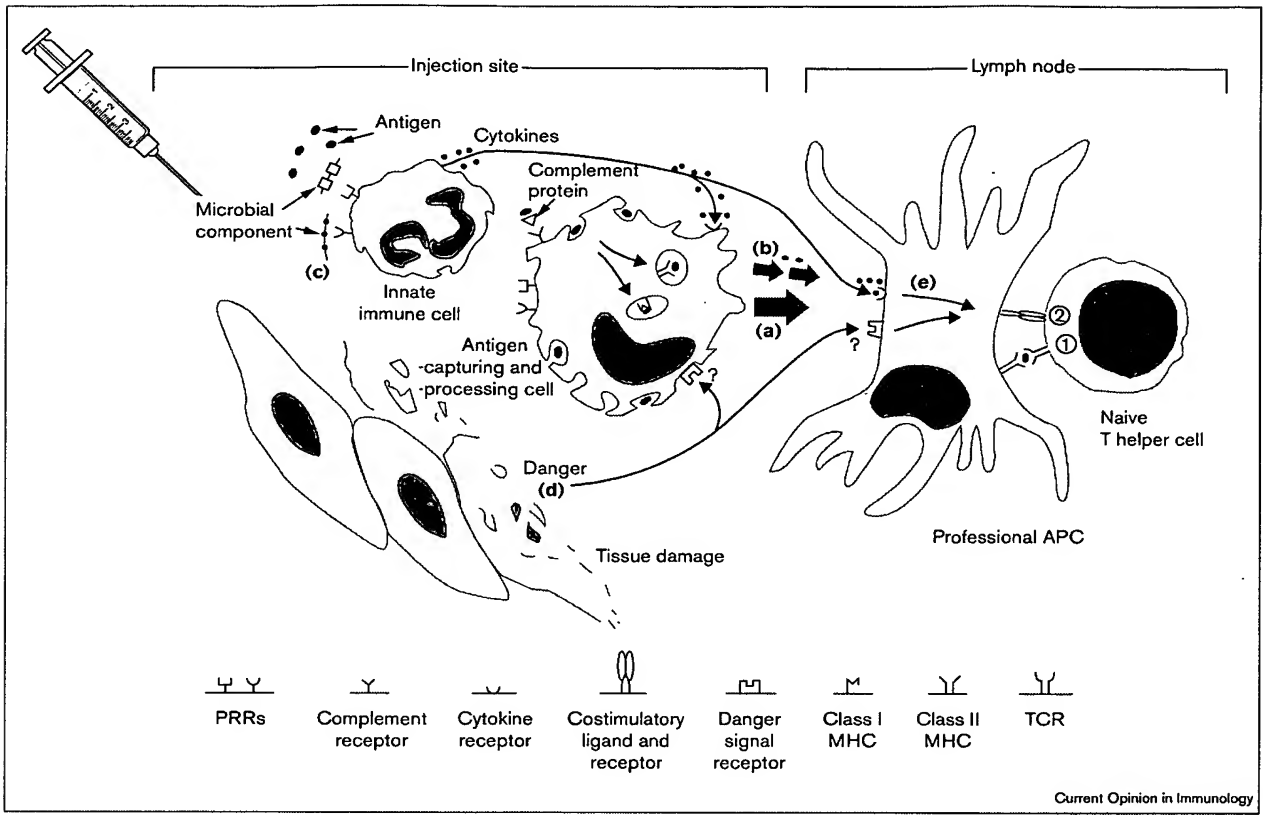
The precise modes of action of adjuvants are poorly understood

The rational design of vaccines initially involves identification of immunological correlates of protection — the immune effector mechanism(s) responsible for protection against disease — and the subsequent selection of an antigen that is able to elicit the desired adaptive response. Once this appropriate antigen has been identified it is essential to deliver it effectively to the host's immune system.

According to current thinking, a productive immune response is defined by the generation of clonally expanded antigen-specific T and/or B cells. This initially requires presentation of the antigen to specific TCRs on naive T cells or to cell-membrane-bound immunoglobulins on B cells. This stimulus is defined as signal 1. In addition, the delivery of costimulatory molecules or cytokines (signal 2) provided by the APC contributes to the priming of T helper cells [1] and their subsequent delivery of antigen-specific help for B cells and cytotoxic T lymphocyte (CTL) effectors. The necessity for signal 2 and the conditions essential for its generation are still controversial [2–4]. Adjuvants typically enhance immunogenicity of co-administered antigens but, surprisingly, little is known about their mode of action. How do these molecules fit in the current concepts?

Traditionally, vaccines come in several forms: live-attenuated, replicating pathogens and non-replicating, inactivated pathogens or their subunits. The latter, non-viable, category is the most safe one but, because of poor or no immunogenicity, often requires adjuvants (*adjuvare* is Latin for "to help") to elicit an adequate immune response. In the absence of adjuvant a lack of responsiveness may occur and naive antigen-specific T cells may recognize the antigen but become tolerized. Adjuvants are defined as a group of structurally heterogeneous compounds, used to evoke or increase an immune response to an antigen [5]. Classically recognized examples include oil emulsions, saponins, aluminium or calcium salts, non-ionic block polymer surfactants, derivatives of lipopolysaccharide (LPS), mycobacteria and many others. Theoretically, each molecule or substance that is able to favor or amplify a particular situation in the cascade of immunological events, ultimately leading to a better immunological response, can be defined as an adjuvant. Obviously, the first step is very important. However the *in vivo* molecular and cellular mechanisms required for the generation of an effective immune response, which depends critically on co-injection of adjuvant, are poorly understood. Moreover the structural requirements of adjuvants are unknown. Adjuvants have therefore been surrounded by obscurity and called "the immunologist's dirty little secret" by Janeway in 1989 [6]. The present overview attempts to classify adjuvants functionally according to at least five recently proposed concepts of immunogenicity (a–e in Table 1 and Figure 1): firstly the geographical concept of immune reactivity and secondly the theory of depot effect, both emphasizing the importance of antigen localization for a period of time after immunization; thirdly the paradigm that adjuvants act as

Figure 1



Visualisation of the essential steps of different concepts of adjuvanticity. **(a)** Facilitation of antigen transport, uptake and presentation by antigen-capturing and -processing cells in the lymph node draining the vaccine injection site. **(b)** Repeated or prolonged release of antigen to lymphoid tissues (depot effect). **(c)** Signalling of PRRs activates innate immune cells to release cytokines necessary for upregulation of costimulatory molecules. **(d)** Danger signals from stressed or damaged tissues alert the antigen-presenting cells to upregulate costimulatory molecules. **(e)** Note that signalling by recombinant cytokines or costimulatory molecules mimics classical adjuvant activity. Steps (c), (d) and (e) allow signal 2 as well as signal 1 from APCs.

signal 0; fourthly the hypothesis that adjuvants induce or act as danger molecules; and finally the role of signal 2 molecules as natural adjuvants. The last three dogmas stress the significance of the activation status of the APCs.

The geographical concept of immune reactivity

It is not known whether an adjuvant exerts its activity at the site of injection or in the local draining lymph node. Naive T cells do not enter nonlymphoid areas of the body efficiently. They rather recirculate between secondary lymphoid organs, such as the spleen and lymph nodes, via the blood and efferent lymphatics. Only memory and effector lymphocytes are able to penetrate nonlymphoid tissues during inflammation [7,8]. According to the recently proposed geographical concept of immune reactivity, the induction of an immune response critically depends on antigen (signal 1) reaching, and being available in, lymphoid organs [2]. Antigen that does not reach the draining lymph nodes is not responded to [2]. In the lymphoid tissue, signal 2 is present in abundant quantities and does not need to be upregulated as long as there is a sufficient load of antigen (signal 1) [9]. Mice that lack lymph nodes are strongly immunocompromised [10]. Thus initiation of immune responses takes place exclusively in lymphoid organs where, in general, initial interactions between antigen-loaded APCs and T cells allow the completion of T cell dependent immune responses [11]. When the passage to the lymph node is prevented by interruption of the afferent lymphatics before the antigen has reached the lymphoid tissue, no immune response develops [12–15].

In view of these considerations, immune responsiveness that is increased or initiated by adjuvants may simply be a result of enhanced translocation of vaccine antigen from the peripheral site of injection towards the draining local lymph node. In this process, antigen-capturing dendritic

Table 1

Classification of adjuvants according to immunological events they induce.

Group	Concept of action	Examples of adjuvants	Key event(s)
a	Facilitation of antigen uptake, transport and presentation by APCs	ISCOMs, Quil A, $Al(OH)_3$, Liposomes, Cochleates, Poly (lactic/glycolic acid)	Antigen localization in the lymph node
b	Depot effect	Oil emulsions, $Al(OH)_3$?, gels, polymer microspheres, non-ionic block copolymers	Prolonged antigen presentation
c	Signal 0	Complement, CpG-rich motifs, LPS (Monophosphoryl lipid A), mycobacteria (muramyl dipeptide), yeast extracts, cholera toxin, ISCOMs?	Signaling of PRRs on innate immune cells
d	Danger signal	Oil-emulsion surface active agents, $Al(OH)_3$, IFNs, hsps, hypoxia, etc.	Tissue destruction/stress
e	Recombinant signal 2	Cytokines, costimulatory molecules	APC polarization, T and B cell help

cells (DCs) are likely to play a central role. DCs are described as "nature's adjuvant" because of their unique ability to turn on naive T cells [16,17]. Immature DCs reside in most tissues as sentinels ready to capture antigen either by receptor-mediated endocytosis or fluid-phase pinocytosis. Upon activation they move from the periphery towards the nearest draining lymph node via afferent lymphatics. DC activation occurs by microbial infections, microbial products (e.g. LPS), TNF-α, IL-1 [18,19], stress and possibly by vaccine adjuvants [20] (Figure 1).

Dupuis *et al.* [21] showed that DCs internalize vaccine antigen and adjuvant MF-59, an oil-in-water emulsion, after intramuscular injection. How DCs know where to go is largely unknown [15]. They are rapidly recruited into sites of tissue injury in response to inoculation with live or inactivated viruses or bacteria, probably as a result of locally produced chemotactic factors [22]. This is often associated with a transient influx of neutrophils. Upon arrival in the lymph nodes, DCs' capacity to capture and process antigen declines whereas their immunostimulatory function is upregulated [15,16]. The antigen remains in the draining lymph node [23].

Fazekas de St Groth [24] emphasized the importance of two different types of DCs with distinct ontogenic origins: myeloid DCs (MDCs), which induce naïve-T-cell priming; and the tolerizing, T-cell-death-programming lymphoid DCs (LDCs). Tolerogenic LDCs contribute to the vast majority of APCs in close contact with naive T cells. Under the influence of adjuvants, MDCs migrate towards T cell zones [24]. The striking influence of the DC subset on the type of immune response was recently demonstrated by comparing immune reactions in mice pretreated with DC-favoring cytokines. MDC-expanding GM-CSF favored Th2 responses whereas mice pretreated with the cytokine Flt3-ligand expanded LDCs, leading to Th1 type responses [25]. The apparent conflicting observation that LDCs can induce both tolerance and Th1 type immunity might be due to the fact that consensus regarding the nomenclature for DC subpopulations and their associated maturation and activation stages has not been reached yet. Nevertheless, according to these data, adjuvants may act by preferentially involving the appropriate type of competent DC — the professional APC [4], able to internalize the antigen — to migrate towards the T cell zone and present antigen to naive T or B cells.

In summary, according to the geographical concept of immune reactivity, antigen in the lymphoid organs is of critical importance for immune responsiveness; antigen location and dose and the timing of presentation are the most essential parameters. Newly introduced antigen remaining outside the lymphoid tissues is not recognized. As a consequence, immunostimulation by adjuvants may result from increased attraction of DCs towards the injection site, increased loading of APCs or increased transport of antigen-loaded APCs towards the lymph nodes (Figure 1; Table 1). According to this concept, continued adjuvanticity — provided by replicating micro-organisms/vectors — is likely to be based on provision of sufficient amounts of antigen to the lymphoid tissue, as long as replication proceeds [9]. In line with this concept is also the well-known dose-dependence of primary immune responses, following administration of non-replicating antigen, and the dependence on virulence-related antigen load for immune reactions to replicating pathogens. The adjuvanticity of lipid matrix-based vaccines — including liposomes, cochleates, micelles, immunostimulating complexes (ISCOMs) and polymer microspheres — is also in conformity with this hypothesis (Table 1). These vaccines are better phagocytised by antigen-presenting mononuclear phagocytic cells and their antigens may often be introduced into the MHC class I processing and presentation pathway [26], a phenomenon normally restricted to cytosolic antigens.

The concept of the depot effect

The immune-stimulatory activity of repository adjuvants has been ascribed to prolonged delivery of antigens (signal 1). As discussed above, the sustained presence of antigen appears important for costimulation-independent immune responses. Preferentially, the antigen should trigger T cells in the

lymph nodes for a sufficient period of time. In particular, antigen retention on follicular DCs within the draining lymph node is responsible for stimulating continued antibody production [27]. Localization of antigens in follicles is greatly enhanced by pre-immunization [28]. Thus, as soon as antigen-specific antibodies have been formed as a result of a primary immune response, the persistence of antigen in the lymph node or at an injection site is likely to be an important factor for the duration of the response [29,30]. Antigen maintenance at the injection site is effectively established by oil-based adjuvants that form a deposit of antigen (Table 1) [14,31]. However, after a while the release rate of antigen from the oil-induced injection granuloma declines and becomes insufficient to maintain optimal antibody responses; as a result, excision of the walled-off injection site no longer affects antibody response formation in the draining lymph node [14]. Aluminium salts were also thought to work by helping form a depot of antigen. However this notion has been challenged by a recent study of Flarend *et al.* [32] who demonstrated that aluminium salts start to dissolve within one hour after injection, leading to resolution of the antigen depot. Persistence of antigen at the injection site, following immunization or chronic infection, may frequently lead to the development of organized tertiary lymphoid tissue, often with T- and B-zone-like domains, in solid organs [7]. These additional lymphoid tissues may contribute substantially to the duration of the immune response before granuloma formation prevents antigen release [28,33].

Adjuvants act as signal 0

Even injection of antigen-specific TCR-transgenic T cells, uniformly expressing an identifiable TCR of known peptide–MHC specificity, in acceptors that receive the cognate antigen gives no full immune response to the antigen but instead results in tolerance [34]. Despite abundant amounts of signal 1, no effective immune response is generated. The proposed two-signal model for lymphocyte activation postulated that upregulation of costimulatory molecules, signal 2, on APCs is a crucial event determining whether a naive antigen-specific T cell becomes immune or tolerant/ignorant [1]. Janeway and co-workers [3,6,35,36] proposed that an essential event preceding signal 2 is the recognition of conserved microbial structures, so-called pathogen-associated microbial patterns (PAMPs), defined as signal 0. These PAMPs, representing the signature of potentially noxious substances [37], are identified by receptors, so-called pathogen-recognition receptors (PRRs), which are constitutively expressed on cells from the innate immune system. In contrast to the clonally expressed hypervariable antigen-specific receptors on T and B cells, generated by random gene-rearrangements, the recognition molecules used by the innate immune cells (PRRs) are encoded by non-rearranging genes that are genetically inflexible and dependent on germline mutations [3,38]. PRRs on innate immune cells have been selected over evolutionary time to recognize microbial structures that are essential for survival of the pathogen but distinct from eukaryotic cell surface structures of the host [37]. Since PRRs are essential for immunogenicity by generating signal 2 on APCs, they direct the cells of the acquired immune system when, where, to what and how to respond [37,39].

Consequently, rather than influencing the quantity or duration of antigen delivered to the immunologic inductive site, the immunostimulatory activity of vaccine adjuvants may critically result from the induction of secondary signals, for example B-7 family receptors and CD40, on APCs via direct or indirect steps. It has been suggested that adjuvants mimic microbial structures and it was postulated that the basis of adjuvanticity could be the recognition of defined microbially derived agents by the phylogenetically ancient PRRs present on accessory cells [6,40,41]. This is likely for adjuvants based on microbial components, like pertussis toxin, mycobacterium-derived muramyl peptides, LPS, lipid A or CpG-rich motifs. The complement component C3d fused to antigen also strongly stimulates antigen-specific antibody responses, as a molecular adjuvant of innate immunity [42]. However, verification of this concept is not available for aluminium-, oil- or saponin-based adjuvants. The selection of recognition receptors during phylogeny may have differed among species as a result of differences in micro-organism exposure. This may explain the species differences in reactivity to particular adjuvants.

According to the signal 0 concept, non-infectious virus-like particles or inactivated pathogens are likely to be recognized by PRRs on innate immune cells since they strongly resemble PAMPs — they have a repetitive, highly organized antigenic structure [43,44]. However, in contrast to live virus, they mostly fail to elicit an effective antibody response when administered without adjuvant [45]. The cage-like structures of ISCOMs may also mimic PAMPs and stimulate immune responses efficiently. However in these nanoparticles the antigen is physically integrated with the adjuvant, *Quillaja saponaria* Molina saponin [46].

In CD40 (ligand)-deficient mice the importance of signal 2 for humoral responses has clearly been demonstrated during viral infection [47,48] or following immunization using complete Freund's adjuvant [49]. However, mice containing disrupted CD40 ligand genes show antigen-specific immunoglobulin class switching to thymus-independent antigen [50]. These mice generated normal, primary, virus-specific CTL responses [47,48]. Also, data from Kündig *et al.* [51] demonstrated that in mice lacking the important costimulatory molecule CD28 (signal 2), the continued presence of signal 1 — resulting from prolonged viral replication or repeated injection of viral peptide — prevents anergy and leads to a functional T cell response. In B7-1 deficient mice, T cell activation to alloantigen was also noted [52]. CD28 deficient mice exhibit strongly impaired humoral and cellular responses against type II collagen, when administered in complete Freund's adjuvant [53]. Evidently, the fact that mice lacking particular costimulatory molecules can mount essentially normal or partial immune responses may be explained by the use of alternative costimulatory pathways by different antigens or adjuvants [52,54,55].

The distinction between non-infectious self and infectious nonself by ancient receptors on innate immune cells can only occur when the ligands and receptors are brought into physical contact. In the case of an immunization it is not known whether this occurs at the injection site, in the draining lymph node or at both sites. During natural infections via mucosal epithelial layers or the skin, the innate immune cells — for example macrophages, mast cells, neutrophils and γδ T cells — are strategically situated at the port of entry of the micro-organisms [37]. However innate immune cells may initially be sparse at frequently used vaccine injection sites, for example the muscle. They might play a more prominent role in the local draining lymph nodes after the antigen is transported by DCs, a crucial factor in the geographical concept of immune reactivity. Investigations of the influence of a water-in-oil emulsion without antigen, inoculated at a different time or place to the antigen, showed that only mice given antigen together with adjuvant at the same site showed significant enhancement of antibody formation [56]. Interestingly, subcutaneous or intraperitoneal application of oil-emulsion adjuvant and concomitant intravenous injection of antigen resulted in enhancement of antigen-specific splenic antibody formation [57,58]. In contrast, subcutaneous injection of adjuvant and antigen at distinct (contralateral) sites did not increase the humoral immune response [56]. Recent studies addressing the role and location of cells at the site of immunization, using surgical ablation experiments, revealed that removal of the vaccinated muscle bundle within 1–10 minutes did not affect primary antibody and CTL responses [59]. This suggests that antigen, but probably also adjuvant, may reach immune induction sites like the lymph node very rapidly after intramuscular application. Hence, for many different adjuvants the critical anatomical site of adjuvanticity remains questionable.

Adjuvants represent or induce danger molecules

According to the 'danger model' of the immune response [4], signals from damaged or stressed cells start an immune response. Antigen-capturing APCs become activated in peripheral tissues only in situations that are dangerous to the host tissues. The so-called danger signal comprises tissue destruction and necrosis and directly evokes expression of costimulatory molecules on the APC. This paradigm advocates that the immune system distinguishes dangerous from harmless instead of self from non-self [4,60,61]. The danger signals also include infection, cell stress, temperature shifts, hypoxia, trauma, mitochondria and heat-shock proteins (hsps; e.g. hsp96); these are signals from damaged or infected cells but not apoptotic cells [62]. Accordingly, an adjuvant could be defined as a danger (-inducing) signal. In a recent extension of this concept it was suggested that damage-induced stress proteins in particular act as chaperons for antigens, thereby increasing the capture and presentation capacities of the APCs [63]. In fact, eukaryotic hsps have been shown to exert adjuvant activity [64,65].

Necrosis of individual muscle fibres and intramuscular oedema in interstitial connective tissue — associated with a marked influx of neutrophils — has indeed been observed within hours after vaccination for a number of adjuvants including aluminium hydroxide, calcium phosphate and mineral oils [66–69]. Thus, the ability to cause danger by inducing local reactions at the injection site may be a property shared by most of the commonly recognized adjuvants. It has been suggested that the immune responses are proportionally related to the tissue damage evoked by the adjuvant. Accordingly, new adjuvants may mimic danger signals. Indeed, Gallucci *et al.* [62] showed that co-administration of ovalbumin with necrotic cells or with freshly isolated and damaged blood vessels increased primary anti-ovalbumin delayed-type-hypersensitivity reactions almost as efficient as complete Freund's adjuvant. However injection of cell debris, obtained by freeze-thawing of allogenic splenocytes, had no adjuvant effect on antibody formation against an inactivated viral antigen (VEJC Schijns, T Jansen, H van Zuilekom, S Cox, unpublished data). This does not refute the danger explanation but raises doubts about its generality or its relative contribution to antibody-forming responses. The adjuvanticity of liposomes also does not easily fit into this concept.

Signal 2 molecules as natural adjuvants

According to the danger model [4] and the concept of immunoregulatory innate immune cells dictating immune responsiveness, the expression of costimulatory molecules on APCs is considered essential for efficient antigen presentation and priming of naive T and B cells [6,40]. Codelivery of costimulatory molecule CD86 (B7-2) has been shown to result in an enhancement of T cell mediated immune responses [70]. B7 transfection of tumor cells that are normally ignored results in their immunogenicity [71]. DNA encoding antigen–CTLA4 fusion proteins also evoked increased humoral and cellular immune responses [72].

A remarkable difference between administration of antigen alone, which results in non- or low-responsiveness, and administration of antigen plus adjuvant, resulting in priming, is the induction of inflammation. Inflammatory cytokines produced by macrophages or innate immune cells at the injection site may be essential communicators of adjuvant activity. The only identified cytokine induced by $Al(OH)_3$, muramyl dipeptides and saponins is the proinflammatory cytokine IL-1 [73]. Different profiles of cytokines have recently been detected in the efferent lymph of lymph nodes from sheep immunized with different adjuvants [74]. ISCOMs have also been shown to evoke proinflammatory cytokines: IL-1, IL-6, IL-12 and TNF-α [46,75]. Based on antibody isotype monitoring, several classic adjuvant formulations have been shown to elicit preferentially a Th1 or a Th2 type response in mice. The mechanism by which the adjuvant induces production of the prototypic initiation molecules, IL-12 or IL-6/IL-4, leading to polarization of T helper cell response is unknown. Selective activation of particular types of innate immune cells or activation of particular receptors on one innate immune cell type is a possibility [76]. These

response-steering stimuli might be referred to as signal 3 [77]. Recently, IL-1 and IL-12 were identified as essential and sufficient signals — together with the TCR (signal 1) and IL-2 (signal 2) — to optimally activate naïve $CD4^+$ and $CD8^+$ TCR-transgenic T cells, respectively [78]. In this study the effects of the cytokines on, or derived from, APCs were eliminated by culturing the T cells with complexes of MHC with protein/peptide antigens that were immobilized on inert latex microspheres.

Obviously, cytokines themselves are being evaluated as adjuvants since they are likely to be the critical communication signals of the more 'classical' adjuvants. Indeed, in an accumulating number of publications it has been demonstrated that exogenous cytokines — either as recombinant proteins, or expressed by recombinant vectors or by naked plasmid DNA — act as vaccine adjuvants [79–81]. These observations strongly suggest that endogenously produced cytokines also act as essential communication signals elicited by traditional adjuvants. However, although IL-6 deficient mice fail to mount a normal inflammatory response to localized tissue damage, they generate a normal systemic inflammation following LPS injection [82]. Similarly, the adjuvant activity of alum and Freund's adjuvant is not diminished in mice genetically defective for IL-6 or TNF-receptor-1 [83]; neither is the intestinal adjuvant activity of cholera toxin impaired in IL-6 deficient mice [84]. In contrast, IL-6 deficient mice are resistant to autoimmune encephalitis that is induced by complete Freund's adjuvant [85]. Similarly, ISCOM-induced responses are normal in IL-4-, IL-6-, IFN-γ-receptor- and iNOS-deficient mice but clearly impaired in $IL\text{-}12^{-/-}$ mice [86]. The redundancy of the cytokine network makes it difficult to ascribe the activity of a particular adjuvant to one or more cytokines. Cytokines crucial for immunogenicity may include the proinflammatory IFN-α/β, TNF-α, IL-1, IL-6, IL-12, IL-15 and IL-18, which influence antigen presentation. Others may act more downstream during clonal expansion and differentiation of T and B cells, with IL-2, IL-4 and IFN-γ as prototypes [87,88]. When dosed and targeted carefully, cytokines or costimulatory molecules may represent ideal natural immunostimulants for rational choices of adjuvants with minimal toxicity and minimal local reactions.

Perspective

Since its early years, vaccine research has been based largely on empiricism and exercise of judgement. The investigations were mainly driven by testing of various antigen preparations and by variations in the choice of the adjuvant, the dose and route of administration, the vaccination schedule and so on without knowledge of protective immune responses. Nevertheless, this trial-and-error approach has resulted in remarkably effective vaccination programs employed in human and veterinary medicine [89].

In the past several years, knowledge about correlates of protection of many infectious diseases has accumulated. Advances in biochemistry and molecular biology have resulted in new and refined approaches for antigen preparation. In addition, there has been tremendous progress in the identification of novel gene products involved in the regulatory pathways governing the immune response. Already, the advent of these new techniques provides the basis for new, more effective approaches to direct immune responses during vaccination. As outlined above, adjuvant activity may be based on the induction of costimulatory activity by non-self microbial structures, by danger signals from stressed tissue or by localized and sustained delivery of antigen according to the geographical concept of immune reactivity. Each of these paradigms emphasizes the necessity of a distinct key process in the immunological cascade. However, certain adjuvants may be classified mechanistically to more than one of these hypotheses. For example, purified microbial components — for example LPS or extracts of *Toxoplasma gondii* — rapidly increase not only the number of antigen-presenting DCs and their migration but also IL-12 production [90]. Hence, adjuvants can immediately determine the nature of the subsequent adaptive immune reaction. Studies challenging the concepts of adjuvant activity, by addressing the above-considered key immunological events at the cellular and/or molecular level, will provide mechanistic knowledge on adjuvant activity and form a basis for improved and new immunostimulants. They may further the design of rational vaccines and contribute to the fundamental understanding of immunogenicity.

In conclusion, according to mutually non-exclusive immunological concepts, adjuvants can be categorized as immunostimulatory helper moieties that superimpose one or more characteristics onto otherwise poorly immunogenic antigen: such characteristics include a typical pattern of foreignness, the capacity to induce inflammation, the ability to damage cells at the antigen injection side and localization/duration in secondary lymphoid tissues.

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